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Cytochemical Localization of Glucose Oxidase in Peroxisomes of *Aspergillus niger*

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Summary. The subcellular localization of glucose oxidase (E.C. 1.1.3.4) in mycelia of *Aspergillus niger* has been investigated using cytochemical staining techniques. Mycelia from fermenter cultures, which produced gluconic acid from glucose, contained elevated levels of glucose oxidase and catalase. Both enzymes were located in microbodies. In addition, when the organism was grown on glucose with methylamine as a nitrogen source, amine oxidase activity was detected in the microbodies. These organelles can therefore be designated as peroxisomes.

Introduction

Glucose oxidase (E.C. 1.1.3.4) catalyzes the oxidation of glucose to D-glucono-δ-lactone with oxygen as an electron acceptor. The enzyme is of considerable industrial importance. It is widely used for the determination of glucose and commercial applications have been found in the desugaring of egg products and in removing oxygen from certain foods and beverages (Ward 1967). Furthermore, glucose oxidase-producing moulds such as *Aspergillus* and *Penicillium* species are used for the biological production of gluconic acid.

Although in vitro other electron acceptors can replace oxygen to a limited extent (Bentley 1963) there can be no doubt that oxygen is the in vivo electron acceptor for glucose oxidase. It is well known, for example, that production of gluconic acid by mould mycelia is strongly dependent on the dissolved oxygen tension. This phenomenon reflects one of the peculiar kinetic properties of glucose oxidase, shared by other hydrogen peroxide producing oxidases, namely a low affinity for oxygen (Gibson et al. 1964). In addition, oxidation of glucose to gluconic acid by glucose oxidase-containing mycelia of *Penicillium notatum* and *Aspergillus niger* is not inhibited by 1 mM KCN (Keilin and Hartree 1948; van Dijken and Veenhuis, unpublished).

As a result of the in vivo operation of glucose oxidase with oxygen as the electron acceptor, hydrogen peroxide is formed in equimolar amounts with gluconic acid. It

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is almost certain that catalase prevents hydrogen peroxide intoxification in cells which produce gluconic acid with glucose oxidase. The enzyme is invariably present in such cells and catalase is a known contaminant of commercial preparations of glucose oxidase from fungi (Pazur and Kleppe 1964; catalogues of manufacturers of glucose oxidase).

In *Aspergillus niger* glucose oxidase is an intracellular enzyme (Pazur 1966). It was therefore of interest to study its subcellular localization, particularly since hydrogen peroxide-producing oxidases are not present in the cytoplasm of eucaryots, but occur in peroxisomes (De Duve and Baudhuin 1966; Masters and Holmes 1977). The results of cytochemical staining experiments presented in this paper show that glucose oxidase is not a cytoplasmic enzyme in *Aspergillus niger* but is immobilized in (catalase containing) peroxisomes.

Materials and Methods

Microorganism and Cultivation

A strain of *Aspergillus niger* producing moderate amounts of glucose oxidase was used throughout this study. The organism was a gift from Dr. J.L. Meers, John and E. Sturge Ltd., Selby, U.K. Cultivation methods were adopted such that growth in pellets was avoided and occurred with a minimum of lysis (as judged from the glucose oxidase activity of supernatants). The fungus was cultivated on a mineral medium containing (per litre) : KH_2PO_4, 1 g; methylamine hydrochloride, 2 g; $MgSO_4 . 7H_2O$, 0.25 g; K_2SO_4, 1 g; $ZnSO_4 . 7H_2O$, 0.03 g; $FeSO_4 . 7H_2O$, 0.03 g and yeast extract, 0.3 g. Before sterilization the pH was adjusted to 5.6 with NaOH. After heat sterilization, sterile glucose was added to a final concentration of 0.3 M 500 ml Erlenmeyer flasks containing 100 ml of the above medium were inoculated with a spore suspension to a final concentration of about 10^7 spores/ml. Incubation was at 30° C on a rotary shaker (300 RPM) for 12 h. This culture was then transferred to a fermenter (Harder et al. 1974) containing 1 L of the above medium. Fermentation was carried out at 30° C under vigorous aeration (2/v/vm) and agitation (800 RPM). The pH was controlled at 5.6 by automatic adjustment with 4N NaOH. When 2/3 of the glucose had been consumed (after 12 - 14 h) the culture was harvested by filtration through a nylon cloth and washed extensively with demineralized water.

Cytochemical Staining Techniques

1. Staining of catalase activity with 3,3'-diaminobenzidine (DAB) and hydrogen peroxide. Cells were fixed in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min at 0° C, washed with 0.1 M cacodylate buffer pH 6.6 and resuspended in an incubation medium containing 0.05% H_2O_2 and 2 mg/ml DAB in 0.1 M cacodylate buffer pH 6.6 Incubation was for 2 h at 37° C. After 1 h the medium was renewed.

2. Staining of glucose and amine oxidase activity with DAB. Cells were fixed in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.0 for 30 min at 0° C washed with

distilled water and resuspended in an incubation medium containing 2 mg/ml DAB and 100 mM glucose in 70 mM potassium phosphate buffer pH 6.6. Incubation was for 1 h at 37° C. During incubation the suspensions were continuously aerated and the medium was renewed every 30 min. In control experiments glutaraldehyde fixed cells were preincubated during 30 min in 70 mM phosphate buffer pH 6.6 containing 50 mM aminotriazole or 1.5 mM KCN and then transferred to the incubation mixture containing the same concentration of inhibitor. Anaerobic incubations were performed by bubbling oxygen-free nitrogen through the solutions during incubation. For the localization of amine oxidase the same procedure was adopted except that glutaraldehyde fixation was for 15 min and glucose was replaced by methylamine hydrochloride (20 mM).

Fixation Techniques

Whole cells were fixed with either 1.5% $KMnO_4$ for 20 min at room temperature or with 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 0° C for 1 h, washed with buffer and postfixed in a mixture of 1% OsO_4 and 2.5% $K_2Cr_2O_7$ in 0.1 M cacodylate buffer pH 7.2 for 45 min. Poststaining was performed in 1% aqueous uranylacetate overnight.

After cytochemical staining cells were postfixed in 1.5% $KMnO_4$ for 20 min and poststained in 1% uranylacetate overnight.

After poststaining, cells were embedded in 1.5% agar, dehydrated in a graded alcohol series and embedded in Spurr's (1969) epoxy resin or Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 electron microscope.

Enzyme Assays

For the preparation of cell-free extracts mycelium was suspended in 50 mM potassium phosphate buffer pH 7.0. The suspensions were sonified at 20 kHz for 5 min at 0-4° C with an MSE 100 W ultrasonic desintegrator. Whole cells and debris were removed by centrifugation (20 min at 20 000 x g). The supernatant was used for enzyme assays. Glucose oxidase and amine oxidase activity in cell-free extracts were determined with a Clark-type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., Ohio, USA) at 30° C. The total volume in the reaction chamber was 3 ml. For glucose oxidase the reaction mixture consisted of 0.1 M citrate/ phosphate buffer pH 5.6 catalase, 400 µg; extract and glucose, 100 mmol. For amine oxidase assays the reaction mixture consisted of 50 mM potassium phosphate buffer pH 7.5; catalase, 400 µg; extract and methylamine, 5 mmol. Initial oxygen uptake rates were taken as a measure for the reaction velocity. Catalase was assayed according to the method of Lück (1963). Enzyme activities are expressed as units per mg cell-free extract protein. For the oxidases one unit corresponds to 0.5 µmol O_2 consumed/min. Catalase units are expressed as ΔE 240/min.

Protein concentration in cell-free extracts were determined by the method of Lowry et al. (1951) with bovin serum albumin as standard.

Table 1. Activities of H_2O_2-producing oxidases and catalase in cell-free extracts of *Aspergillus niger*, grown on glucose and methylamine in shake flasks and aerated fermenters, 14 h after inoculation. For growth conditions see Materials and Methods. Activities are given in units/mg protein

Growth conditions	Glucose oxidase	Amine oxidase	Catalase
Shake flask culture	0 – 0.01	0.06 – 0.09	3 – 5
Fermenter culture	2.55 – 4.70	0.01 – 0.02	7 – 10

Results

Oxidase Activities and Ultrastructure of Aspergillus niger *During Growth on Glucose and Methylamine*

Extracts of cells of *Aspergillus niger* grown in shake flasks on glucose with methylamine as a nitrogen source contained amine oxidase and catalase activities but only low or undetectable amounts of glucose oxidase (Table 1). An ultrastructural analysis of such cells revealed the presence of a small number of large microbodies, besides the usual cell organelles such as a nucleus, mitochondria, ER and vacuole (s) (Fig. 1). The microbodies were 0.2 - 0.6 μ in diameter and surrounded by a unit membrane of approximately 70 Å thick. After transfer of such cells to well-aerated fermenters the growth rate decreased and a drastic increase in glucose oxidase activity was observed (Table 1) along with the excretion of gluconic acid, a phenomenon not observed in shake flasks. Significant changes in ultrastructure occurred. The number of mitochondrial profiles increased and up to 4 nuclei were observed per cell. Also the number and dimensions of the microbodies had increased. Generally 2 to 5 organelles were observed in thin sections of $KMnO_4$ - fixed cells, irregular of shape, up to 1.7 μm in diameter (Fig. 2). The organelles were scattered throughout the cytoplasm although small clusters of a large and one or two small organelles were also observed. As in the inoculum cells, close associations of microbodies with mitochondria and strands of ERwere evident (Figs. 1 and 3). Sections of glutaraldehyde $OsO_4/K_2Cr_2O_7$-fixed cells revealed the absence of crystalline inclusions in the matrix of the microbodies (Fig. 4).

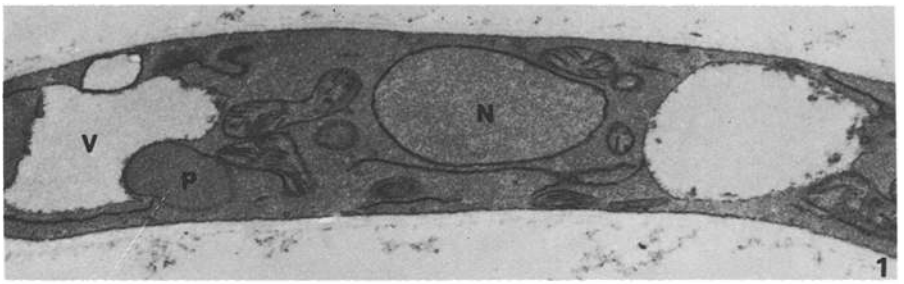
Cytochemical Staining of Catalase and Oxidases in Aspergillus niger

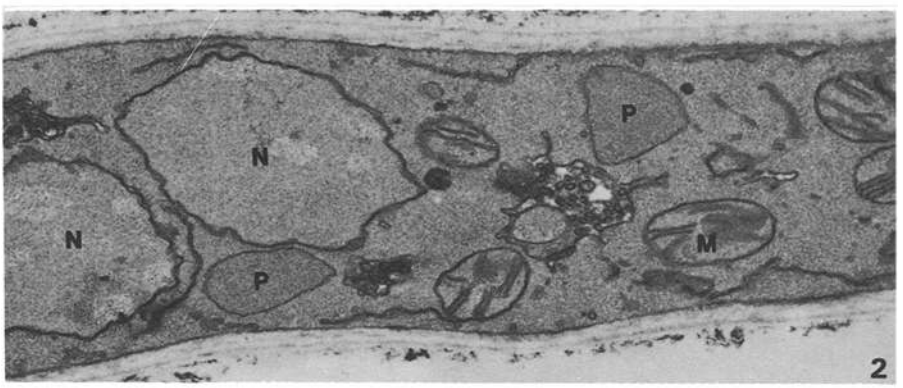
The presence of catalase activity in the microbodies of cells of *Aspergillus niger* was apparent after incubation of glutaraldehyde-fixed cells with DAB and exogenous H_2O_2 (Fig. 8). Incubations of glutaraldehyde-fixed cells from fermenter cultures with DAB and glucose as the oxidase substrate also resulted in positively stained microbodies, indicating the presence of glucose oxidase activity in these organelles (Figs. 5 and 6). In addition, also amine oxidase activity was located in these organelles as was

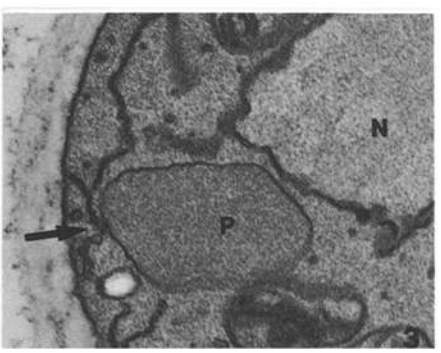
demonstrated after incubations with DAB and methylamine as the oxidase substrate (Fig. 7). In contrast to amine oxidase, the activity of which could be stained in cells from shake flasks as well as from fermenter cultures, no or only a faint staining was observed in the microbodies of cells grown in shake flasks when stained for glucose oxidase, even after prolonged incubation. This is in accordance with the results of enzyme assays with cell-free extracts (Table1) which showed that cells grown in shake flasks contained only very low or undetectable amounts of glucose oxidase. Control experiments, performed in the presence of aminotriazole, to inhibit catalase activity, showed unstained microbodies after incubations with DAB and glucose or methylamine as well as after incubation with DAB and H_2O_2 (Fig. 9). In addition, anaerobic incubations with DAB in the presence of an oxidase substrate, also resulted in unstained microbodies (Fig. 10). In all incubations with DAB an intense staining of the mitochondria was also observed (Figs. 5-10). Since the mitochondrial staining was independent on the presence of any additional substrate, it seems likely that this is due to a reaction of mitochondrial peroxidases with DAB (Hoffman et al. 1970).

Discussion

Cells of *Aspergillus niger* grown under conditions that require the synthesis of hydrogen peroxide producing oxidases (i.e., glucose oxidase and amine oxidase) contained organelles which, judged by their morphological appearance are microbodies: organelles with a finely granular matrix of moderate electron density surrounded by a unit membrane, which is thinner than the plasma membrane (Masters and Holmes 1977). Microbodies have been shown to occur in all eucaryots investigated so far, including yeasts and filamentous fungi (Avers 1971; van Dijken et al. 1975; Sahm et al. 1975; Fukui at al. 1975; Maxwell et al. 1977). According to the definition of de Duve and Baudhuin (1966) microbodies which contain H_2O_2-producing oxidase and catalase are peroxisomes. The microbodies which specifically develop in methanol-utilizing yeasts have recently been shown to be peroxisomes on the basis of their enzymatic content (Fukui et al. 1975; Roggenkamp et al. 1975; Veenhuis et al. 1976). In filamentous fungi, however, the occurrence of H_2O_2 producing oxidases in microbodies has so far not been substantiated (Maxwell et al. 1977). The finding that *A. niger* grown in well-aerated fermenter cultures on glucose with methylamine as the nitrogen source contained microbodies with glucose oxidase, amine oxidase and catalase activity (Figs. 6-8) provides the first example of the cytochemical demonstration of peroxisomes in filamentous fungi. In this respect it should be noted that data on the presence of catalase in higher fungi are scarce; only a few biochemical studies have indicated the presence of catalase in microbodies of filamentous fungi; also the application of the DAB technique to locate this enzyme has not been generally successful (Maxwell et al. 1977). The presence of glucose oxidase in peroxisomes of *Aspergillus niger* adds another enzyme to the growing list of H_2O_2 producing oxidases which have been shown to be present in peroxisomes of various organisms. Among these is amine oxidase (Yamada et al. 1965a,b) an enzyme which has been shown to be involved in methylamine utilization by the yeasts *Hansenula polymorpha* and *Candida utilis* (Zwart et al. 1980).







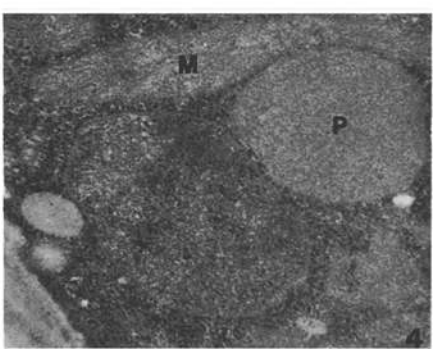
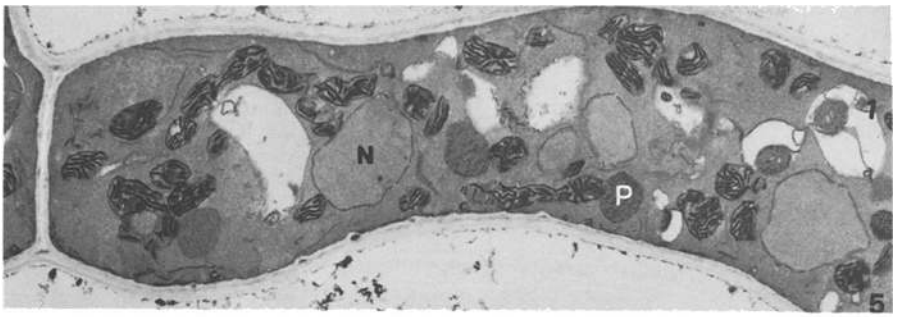
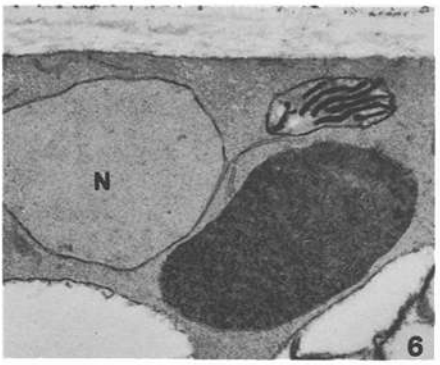


Fig. 1. Survey of a cell from the shake flask culture (24 000 x)
Fig. 2. Survey of a cell from the fermenter culture (25 000 x)
Fig. 3. Detail, to demonstrate the close association with ER *(arrow)* and mitochondrion with th peroxisome (36 000 x)
Fig. 4. Detail of a glutaraldehyde-OsO_4/$K_2Cr_2O_7$fixed cell to demonstrate the homogeneous structure of the peroxisomal matrix (31 500 x)
Fig. 5. Survey of a cell, incubated with DAB and glucose, showing positively stained peroxisom (10 500 x)
Fig. 6. Detail of a cell after incubation with DAB and glucose (23 500 x)
Fig. 7. Detail of a cell after incubation with DAB and methylamine showing a positively stained peroxisome (33 500 x)
Fig. 8. Survey of a cell, incubated with DAB and H_2O_2. The peroxisomes are well-stained (10 000 x)
Fig. 9. Detail of a cell incubated with DAB and glucose in the presence of aminotriazole. The peroxisome remained unstained (31 500 x)
Fig. 10. Detail of a cell, after anaerobic incubation with DAB and glucose, demonstrating an unstained peroxisome (29 000 x)

Abbreviations: *M* = mitochondria, *N* = nucleus, *P* = peroxisome, *V* = vacuole, *DAB* = 3,3'-diaminobenzidine.
The micrographs are taken of $KMnO_4$-fixed cells from the fermenter culture unless otherwise indicated


N
P
5


N
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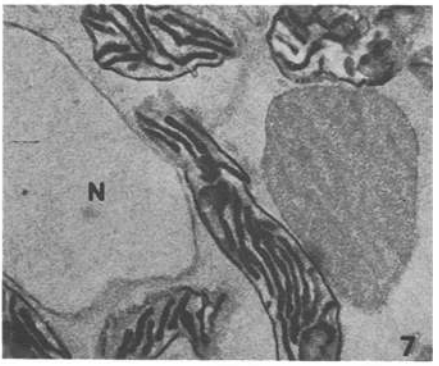
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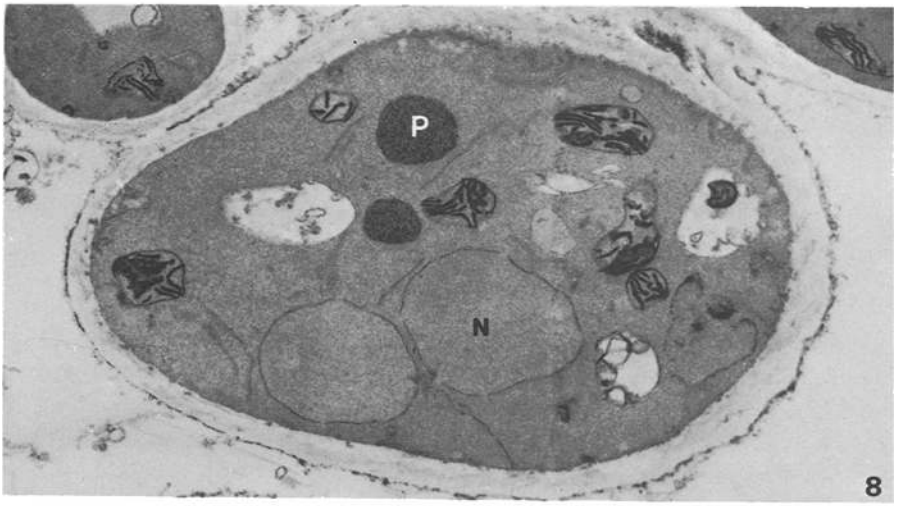
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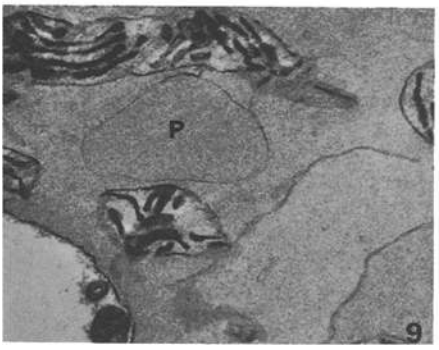
P
9


P
10

In *Aspergillus* glucose oxidase is an intracellular enzyme (Pazur 1966) (Figs. 5 and 6 but the enzyme from *Penicillium* species generally has been isolated from the cultur fluid of these fungi (Kusai et al. 1960). It remains to be investigated whether in thes organisms glucose oxidase is also located in peroxisomes. If so, extracellular glucose oxidase must result from cell-lysis.

Immobilized glucose oxidase has received great interest as a catalyst for preparative or analytical purposes – either as free enzyme or as an immobilized system (Buchhc and Gödelmann 1978). It would therefore be of interest to investigate the propertie of immobilized peroxisomes containing glucose oxidase. Pioneering studies of Japanese workers with alcohol oxidase-containing peroxisomes (Tanaka et al. 1978) have opened interesting possibilities in this respect.

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